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THE DEGRADATION OF FORMONONETIN AND DAIDZEIN IN *CICER ARIETINUM* AND *PHASEOLUS AUREUS**

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Abstract—To investigate the fate of isoflavones in *Cicer arietinum L.* and *Phaseolus aureus Roxb.*, experiments with formononetin-(methyl-^{14}C) and daidzein-(4-^{14}C) have been carried out. Using intact plants and separated or sliced plant parts extensive degradation of the substrates in both plants was observed showing catabolic reactions to be involved. The degree of assimilation observed is discussed in relation to the theoretically expected turnover. Data are presented that rhizosphere microorganisms contributed to the observed degradation. Experiments with sterile *Phaseolus aureus* plants revealed significant differences in the metabolism of daidzein and formononetin.

INTRODUCTION

PREVIOUS communications from this laboratory[1,2] presented evidence that formononetin (I) in *Cicer arietinum* L. as well as daidzein (7,4'-dihydroxyisoflavone) (II) and coumestrol (III) in *Phaseolus aureus* Roxb. are not metabolically inactive end products but are further metabolized, with biological half-lives in the order of 50 hr. Further experiments with *C. arietinum* revealed that on approximately the eighth day after germination the amount of formononetin had reached a steady-state level characterized by comparable rates of biosynthesis and turnover.[3] In the case of biochanin A (IV) in *C. arietinum* however, rapid synthesis occurred in young plants only between the third and the ninth day after germination and then production gradually decreased with no comparable turnover.

These findings lend further support to the hypothesis that accumulation of secondary plant products is regulated by the induction or repression of enzyme systems involved in the formation or metabolism of such plant products.[4] From our earlier observations, we could draw no conclusions whether anabolic or catabolic processes are involved. To further investigate the turnover of the above mentioned compounds, formononetin-methyl-^{14}C[5-7] (I) and daidzein-4-^{14}C[8-10] (II) were synthesized and fed to *C. arietinum* and *P. aureus*. This paper describes various experiments designed to determine the fate of isoflavones in these plants.

* Part III in the series "Metabolism of aromatic plant constituents".

[1] W. BARZ, *Z. Naturforsch.* **24b**, 234 (1969).
[2] W. BARZ and B. ROTH-LAUTERBACH, *Z. Naturforsch.* **24b**, 638 (1969).
[3] H. GRISEBACH and W. BARZ, *Naturwiss.* **56**, 538 (1969).
[4] M. H. ZENK, *Ber. Deut. Botan. Ges.* **80**, 573 (1967).
[5] L. JURD, *J. Am. Chem. Soc.* **80**, 5531 (1958).
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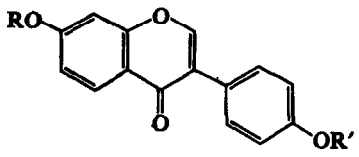
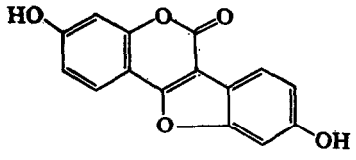
[7] E. WALZ, *Liebigs Ann. Chem.* **489**, 118 (1931).
[8] L. YODER, E. CHENG and W. BURROUGH, *Proc. Iowa Acad. Sci.* **61**, 271 (1954).
[9] P. M. DEWICK, W. BARZ and H. GRISEBACH, *Phytochem.* **9**, 775 (1970).
[10] R. N. JYER, K. H. SHAH and K. VENKATARAMAN, *Proc. Indian Acad. Sci.* **33A**, 116 (51).



(I) R = H; R′ = CH_3
(II) R = R′ = H
(V) R = —$CH_2C_6H_5$; R′ = H
(VI) R = R′ = —$COCH_3$
(VII) R = —$CH_2C_6H_5$; R′ = —$COCH_3$
(VIII) R = —$CH_2C_6H_5$; R = CH_3



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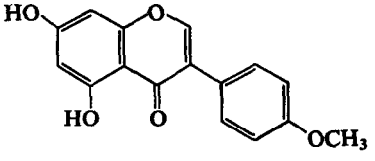

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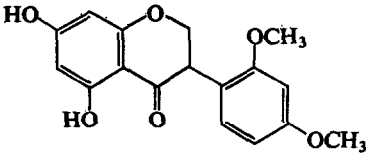

(IX)

RESULTS

Feeding Experiments with Intact Plants

In a first series of experiments, formononetin-(methyl-^{14}C) (·07 μC) was root-fed to 9-day-old hydroponically grown plants[2] of *Cicer arietinum* and *Phaseolus aureus*. Mung bean plants have not been found so far to contain formononetin or other methylated flavonoids. They were, however, included in the experiments in order to obtain an indication as to variation in isoflavonoid metabolism. Assuming that the applied substrate can freely enter the metabolically active isoflavone pool and that the biological half-life of (I) is 48–50 hr,[1] a 24-hr feeding period was chosen. One could then expect that *ca.* 25% of the substrate taken up by the plants should be metabolized.

The plants were fractionated according to the scheme outlined in Fig. 1, which also shows the distribution of label in both plants. By dilution analysis, it was demonstrated that only 8·5 and 6·0%, respectively, of the absorbed formononetin-(methyl-^{14}C) could be reisolated. This fact, together with the randomization of radioactivity between label in all fractions obtained and the high percentage of $^{14}CO_2$ evolved led us to assume that strong demethylating activity might be present in both plants. By chromatographing and counting aliquots of the light petroleum extract[11] and of the three ether fractions it could be shown that substantial radioactivity had been incorporated into the xanthophyll, lutein, and into several phenolic compounds. The almost complete assimilation of formononetin and the high production of $^{14}CO_2$ is in contrast to the previously determined rate of isoflavone turnover in these plants[1] considering the duration of the experiment. Possible explanations are that the higher rate of degradation might be due either to unspecific oxidations[12] of substrate by tissue not normally involved in isoflavone turnover or to degradative processes by rhizosphere micro-organisms.

[11] A. HAGER and T. MEYER-BERTENRATH, *Planta* **69**, 198 (1966).
[12] D. NEUMANN and K. H. TSCHOEPE, *Flora Jena* **156**, 521 (1966).

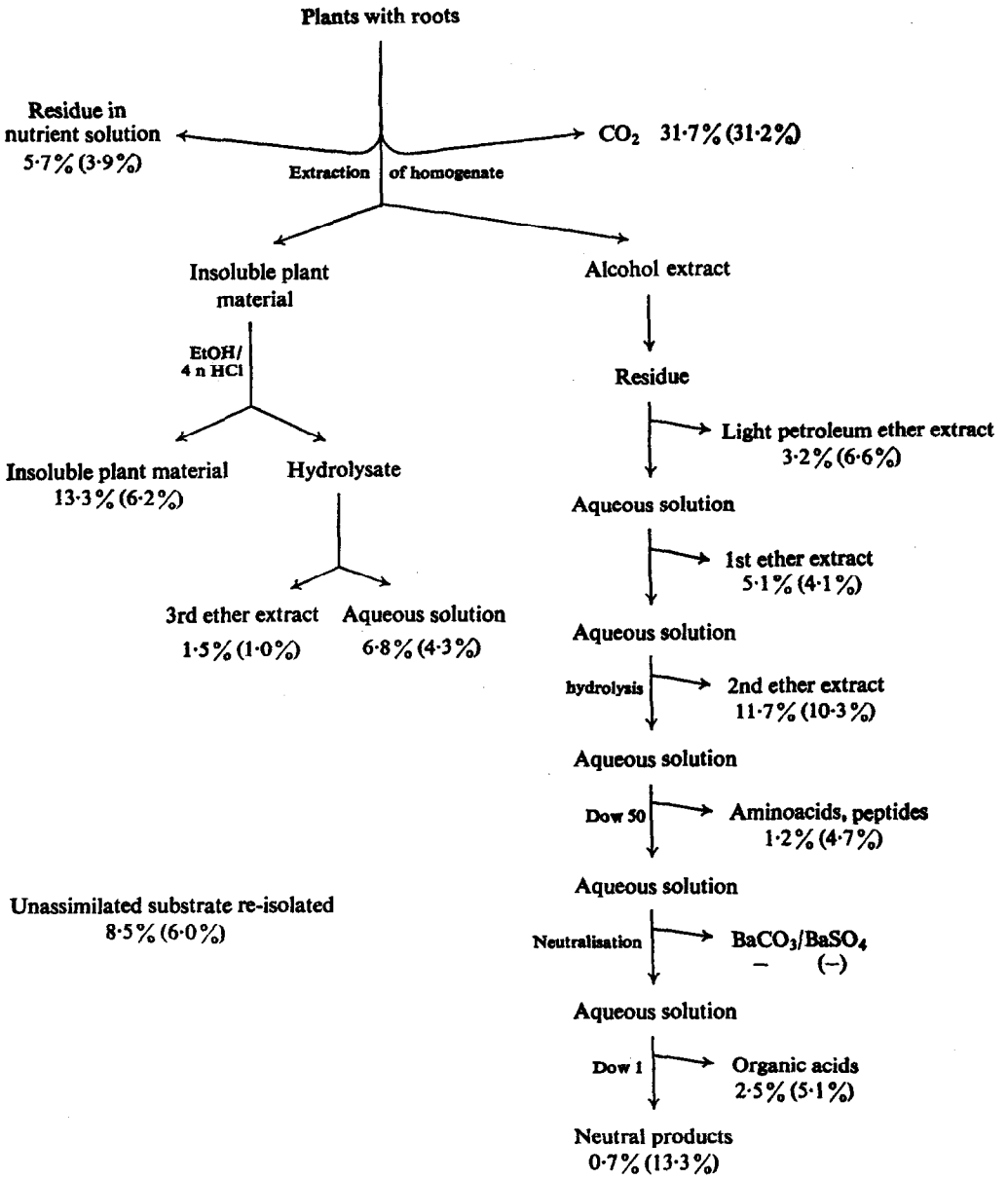

FIG. 1. DISTRIBUTION OF RADIOACTIVITY IN *Cicer arietinum* AND (IN PARENTHESES) *Phaseolus aureus* Roxb. AFTER FEEDING OF FORMONONETIN-(METHYL-^{14}C).

Feeding Experiments with Plant Slices

To test the first-mentioned possibility, separate intact roots or stems and thinly sliced (3–4 mm thick) root or green parts (stems and leaves) of *C. arietinum* were separately incubated with formononetin-(methyl-^{14}C) with efficient aeration. This should ensure better

[13] M. H. ZENK, *Z. Pflanzenphysiol.* **53**, 404 (1965).
[14] H. GRISEBACH and G. BRANDNER, *Experientia* **18**, 400 (1962).

penetration and uptake[13] and decrease the degree of unspecific and irreversible binding of fed phenols to insoluble polymeric structures.[14]

After incubation for 24 hr the plant material was fractionated according to the scheme outlined previously (Fig. 1). The results obtained are summarized in Table 1.

TABLE 1. DISTRIBUTION OF RADIOACTIVITY WHEN FORMONONETIN-(METHYL-^{14}C) IS FED TO SEPARATED OR SLICED PARTS OF *Cicer arietinum*

Fraction \ Plant part	Intact green parts	Sliced green parts	Intact roots	Sliced roots
% Residue in nutrient solution	61·3	33·1	13·3	11·0
% CO_2	1·0	9·8	29·4	35·5
% Insoluble plant material	3·5	3·6	13·6	26·9
% Light petrol extract	6·8	8·3	2·4	0·8
% 1st ether extract	14·7	40·6	9·8	15·7
% 2nd ether extract	9·6	2·1	12·1	6·5
% Amino acids				
organic acids	5·1	0·7	6·8	2·9
neutral products				
% Total	102·0	98·2	87·4	99·3
% Unassimilated substrate re-isolated	42·5	60·4	13·1	22·1

In the experiments with either intact or sliced roots, we again observed a relatively high production (*ca.* 30%) of $^{14}CO_2$ and label was again widely distributed in all fractions obtained. Though formononetin was found in the fractions containing the free and glycosidated phenols—only 13 and 22%, was re-isolated from the intact or sliced root tissue respectively.

When formononetin was absorbed by the intact stems, on the other hand, a very much smaller (1·0%)$^{14}CO_2$ production was observed. Although appreciable randomization of label was found in the phenolic and the non-phenolic fractions, 42·5% of the absorbed formononetin was re-isolated. In the experiment with the sliced leaves, 60·4% of formononetin was not assimilated. Although there was a somewhat greater production of $^{14}CO_2$, the overall distribution of label agreed with that expected if the formononetin was catabolically metabolized.

While we had found considerable labelling of carotenoids and xanthophylls in the experiments with the intact plants mentioned earlier (Fig. 1), significantly less radioactivity appeared in these compounds when separated leaves were used. Root-feeding of intact *Cicer*-plants, however, resulted in significant labelling of carotenoid compounds. This lead us to assume that in the roots it was $^{14}CO_2$, formed from the labelled isoflavone, that entered the plants metabolism via CO_2-fixation and was incorporated into the carotenoid fraction. This is corroborated by the observation that when intact *Cicer arietinum* plants are fed with comparable amounts of $H^{14}CO_3'$ (1,5 μc) to the amount of formononetin-^{14}C given above, similar high specific activities are found in the carotenoids. Table 2 shows the distribution of label in the main carotenoid constituents of *Cicer arietinum* which had been separated as described by Egger[15] and identified by their absorption spectra.[16]

It was earlier observed[17] that liberated *O*-methyl groups can be re-used via the C_1-pool. Therefore biochanin A (IV) and homoferreirin (IX) were isolated from the phenolic fractions

[15] K. EGGER, *Phytochem.* **4**, 609 (1965).
[16] A. HAGER and T. MEYER-BERTENRATH, *Planta* **76**, 149 (1967).
[17] W. BARZ and H. GRISEBACH, *Z. Naturforsch.* **22b**, 627 (1967).

TABLE 2. SPECIFIC RADIOACTIVITY OF CAROTENOIDS ISOLATED FROM *Cicer arietinum* AFTER APPLICATION OF EITHER FORMONONETIN-(METHYL-^{14}C) OR $H^{14}CO_3'$

Experiment (substrate)	Activity (cpm/μmole × 10^{-6})			
	β-Carotene	Lutein	Violaxanthin	Neoxanthin
Intact root-fed *C. arietinum* (formononetin-methyl-^{14}C)	76·3	6·61	80·0	85·7
Green parts of *C. arietinum* (formononetin-methyl-^{14}C)	0·21	0·034	1·0	0·83
Intact *C. arietinum* ($H^{14}CO_3'$)	8·2	7·55	21·0	75·0

of *C. arietinum* in the experiments described above (Fig. 1 and Table 1) in order to examine the extent of re-methylation processes. Biochanin A was found both in the roots and the green parts, while IX could only be isolated from the root extracts. The results in Table 3 demonstrate that all samples proved to give radioactive biochanin A. However, the highest radioactivity was obtained from the experiment using the intact green parts.

TABLE 3. SPECIFIC AND TOTAL RADIOACTIVITY OF BIOCHANIN A AND HOMOFERREIRIN ISOLATED FROM *Cicer arietinum* AFTER FEEDING FORMONONETIN-(METHYL-^{14}C) AND $H^{14}C_3'$

Compound isolated	Plant part used: Intact plants	Green parts	Sliced green parts	Intact roots	Sliced roots	Intact plants fed $H_{14}CO_3'$
Biochanin A (IV)						
Sp. act. (cpm/mmole) × 10^{-4}	4·8	100	4·5	3·2	14	(0·9)
Total act. (cpm)	84	836	132	194	414	(8)
Homoferreirin (IX)						
Sp. act. (cpm/mmole) × 10^{-4}	28·0	—	—	56·0	60·0	(0·01)
Total act. (cpm)	441	—	—	582	630	(0)

A comparison of the data from Tables 2 and 3 leads to the conclusion that feeding of intact plants with either formononetin or $H^{14}CO_3'$ results in significant labelling of carotenoids with little or no radioactivity in other isoflavonoids, while incubation of intact green parts with the isoflavone shows the opposite pattern of labelling.

Evidence for Rhizosphere Microorganisms

The conflicting data presented so far led us to test the roots and the green parts of both our hydroponically grown *Cicer arietinum* and *Phaseolus aureus* plants for the presence of isoflavone-metabolizing microorganisms.

Microorganisms which could be washed off either the roots or the green parts of both plants were separately incubated with formononetin-(methyl-^{14}C). While all preparations from both roots and stems contained micro-organisms as indicated both by microscopic inspection and plating on nutritional agar, only the microbial suspension isolated from the roots completely degraded formononetin-(methyl-^{14}C) within 24 hr. In this case 84–90 per cent of the applied radioactivity was liberated as $^{14}CO_2$. The microorganisms isolated from the green parts produced only negligible amounts of $^{14}CO_2$ (0·01 %), practically all the formononetin being left intact. These results strongly suggest that rhizosphere microorganisms are

mainly responsible for the degradation of formononetin in the above experiments with the roots or whole root-fed plants. The high production of $^{14}CO_2$ in these cases is presumably partly due to microbial oxidation of formononetin-(methyl-^{14}C), and the results in Figure 1 and Table 1 suggested that part of the liberated $^{14}CO_2$ enters the plant via some CO_2-fixation mechanism.

Experiments with Sterile Mung Bean Plants

The isolation of an isoflavone-degrading microorganism from the roots of *Cicer arietinum* and *Phaseolus aureus* as described in a following communication[18] necessitated the use of aseptically grown plants for further feeding experiments. Small batches of ethanol-washed seeds of *Phaseolus aureus* were rigorously surface sterilized with hypochlorite solution, repeatedly washed with water and grown under sterile conditions. The seeds were kept individually in small vials placed in larger test tubes with a layer of nutritional agar at the bottom. Thus it was possible to inspect each plant for microbial contamination prior to use.

The results of experiments with formononetin-(methyl-^{14}C) and daidzein-(4-^{14}C) using these intact sterile plants are shown in Table 4. While in the experiment with the non-sterile

TABLE 4. DISTRIBUTION OF RADIOACTIVITY IN ASEPTICALLY GROWN *Phaseolus aureus* PLANTS AFTER FEEDING OF LABELLED ISOFLAVONES

Substrate Fraction	Formononetin-(methyl-^{14}C) %	Daidzein-(4-^{14}C) %
Residue in nutrient solution	32·1	5·7
CO_2	0·5	1·4
Petrol-ether extract	1·4	5·6
1st ether extract	61·6	6·9
2nd ether extract	2·9	3·6
Amino acids	0·44	1·2
Organic acids, neutral products	0·01	0·4
Insoluble plant material	3·0	71·4
Ether soluble material liberated from insoluble plant material	—	4·4
Amino acids liberated from insoluble plant material	—	6·3
Carbohydrates, organic acids from insoluble plant material	—	0·7
"Lignin"	—	60·0
Total	101·95	96·2
Unassimilated substrate reisolated	95·0	8·5

mung bean plants only 6·0% unassimilated formononetin was observed (Fig. 1), the sterile plants produced only negligible amounts of $^{14}CO_2$ or other nonphenolic radioactive products. Approximately 95 per cent of the substrate which had been taken up by the plants was reisolated after 24 hr. These data clearly show the contribution of the rhizosphere microorganisms to isoflavonoid degradation in the experiment with the non-sterile plants and also indicate that *P. aureus*, apart from glycosidases, does not possess enzymes for the metabolism of the methylated isoflavone. This distinct substrate specificity is further indicated by the results obtained with daidzein-(4-^{14}C). In this case the applied isoflavone was rapidly and extensively assimilated, being incorporated into lipid material, phenols, amino acids, organic acids and

[18] W. BARZ, *Phytochem.* 9, 1745 (1970).

carbohydrates. A major portion of the substrate was incorporated into the ethanol-insoluble plant material which upon acid hydrolysis released three radioactive phenolic compounds. One was identified as daidzein and the other two are more polar compounds exhibiting an intensive greenish-blue fluorescence when exposed to ammonia fumes. A substantial part of the radioactivity cannot be chemically differentiated from lignin or unhydrolysable polymeric phenolic material. In the fraction of the free phenols, coumestrol (III), was again shown to be radioactive thus giving additional proof of the earlier established biogenetic relationship between isoflavones and coumestanes.[9,19] This experiment with daidzein-(4-^{14}C) again indicates that the observed turnover of isoflavones[1,2] can be explained by assuming catabolic pathways to be operating.

DISCUSSION

The experiments reported in this paper were designed to investigate the general fate of isoflavones and to show, that in contrast to the traditional view,[20] these compounds are not metabolically inactive end products. To distinguish between anabolic[21] and catabolic[4] pathways, we have not only tried to determine the overall quantitative assimilation, but, presuming free transport of the phenols into the reactive pools have assumed one should be able to calculate roughly the percentage of the metabolized products from the known biological half-lives[1,2] of turnover and the duration of the experiment. Taking the results presented in Fig. 1 and Table 1, this approach seems to have been helpful, though steady-state kinetics in this field are far more complicated than our present knowledge.

The results of the experiments with the root-fed plants (Fig. 1) and the separated or sliced roots (Table 1) showed extensive degradation of formononetin-(methyl-^{14}C). However, the extensive assimilation of the methyl-labelled isoflavone in the experiments with the roots must partly be explained by participation of rhizosphere microorganisms because of the finding that there is complete degradation of formononetin-(methyl-^{14}C) using microbial preparations washed off the roots of our experimental plants. The rhizosphere microorganisms obviously degraded formononetin-(methyl-^{14}C) to $^{14}CO_2$ part of which re-entered the plant via fixation processes and led to the strong labelling of carotenoid compounds (Table 2). The significant extent of radioactivity in carotenoids in the experiments with $H^{14}CO_3'$ and the reduction in activity when plants without roots were used with formononetin-(methyl- ^{14}C) (Table 2) can be taken as a proof for this assumption.

Unequivocal proof for the contribution of microorganisms was obtained by a comparison of the two experiments with non-sterile and sterile mung bean plants. Sterile plants degraded very little formononetin (Table 4) so that the complete assimilation observed with non-sterile plants (Fig. 1) clearly shows the contribution of microbes located at the roots. However, the assimilation of formononetin-(methyl-^{14}C) observed in the experiments with the intact or sliced green parts of *C. arietinum* (Table 1) cannot be explained by assuming microbial contaminations, because the leaf microbial contaminants did not degrade formononetin to any measurable extent. In addition, in these last two cases, the extent of assimilation was quite close to our theoretical expectations based on the degree of turnover.

The isolation of labelled samples of biochanin A (IV) and homoferreirin (IX) from *C. arientinum* (Table 3) again[17] indicates that methyl-ether groups removed from phenols can

[19] W. Barz and H. Grisebach, *Z. Naturforsch.* **21b**, 1113 (1966).
[20] K. Mothes, *Experientia* **25**, 225 (1969).
[21] J. W. Fairbairn and S. El-Masry, *Phytochem.* **6**, 499 (1967); **7**, 181 (1968).

enter the C_1-pool and be reused for further methylation reactions. It is suggested that the labelling of IV and IX does not originate from $^{14}CO_2$ via photosynthesis, because under our experimental conditions photosynthesis did not lead to labelled isoflavones.

Altogether these results strongly indicate the absolute necessity to test the experimental plants for microbial contamination, especially when experiments with roots are carried out. This seems to be of great importance in all kinds of feeding experiments.[9]

Our results with formononetin suggest that anabolic pathways or extensive binding of this isoflavone to membranes or other polymeric structures such as lignin are not involved in the observed assimilation of formononetin in *C. arietinum*. The very small degree of incorporation of label into any insoluble material (Table 1) is sufficient proof for this.

The results of feeding experiments with daidzein and formononetin to sterile mung bean (Table 4) clearly show a significant difference between these two isoflavones. Although *P. aureus* can degrade daidzein, enzymes for assimilating the methyl ether seem to be absent showing the distinct selectivity of the enzymes involved in daidzein degradation. The results can be taken as additional proof that daidzein is not a metabolic end product as assumed by Miller.[22] The extensive binding of daidzein to insoluble material (Table 4 "lignin") may be due to the presence of polyphenol oxidases in the more woody tissue such as roots and stems where rather large amounts of polymeric leucoanthocyanins and condensed phenols are formed.[23] Experiments with leaves only[24] show that this process seems to be rather specific for root tissue. This is further indicated by the results in Table 1 where such binding to ethanol-insoluble root tissue was found much higher than in the experiments with green tissue.

EXPERIMENTAL

Formononetin-(methyl-^{14}C)

Carefully dried daidzein-diacetate[7] (300 mg) was refluxed for 20 hr with anhydrous $C_6H_5CH_2Cl$ (3·0 ml), KI (400 mg) and anhydrous K_2CO_3 (1·0 g) in dry acetone (20 ml). After filtration and evaporation of the solvent, excess reagent was removed with light petroleum and the residue repeatedly crystallized from MeOH. Daidzein-7-*O*-benzyl-ether-4′-acetate formed colourless needles, m.p. 173°. U.v. spectrum: λ^{max}_{MeOH}+ 228^{i}, 250, 297, 308^{i} nm, unaffected by sodium acetate; $\lambda^{max}_{0.002_{NaOMe}}$ 240, 248, 258, 280^{i} nm. I.r. spectrum: ν CO 1742, 1637 cm^{-1}.

Daidzein-7-*O*-benzylether-4′-acetate when treated under reflux with conc. HCl (1·2 ml) in 30 ml acetone for 1 hr afforded daidzein-7-*O*-benzylether as white prisms from methanol/water, m.p. 169–170°. U.v. spectrum: λ^{max}_{MeOH} 232, 249, 260, 308 nm unaffected by sodium-acetate; $\lambda^{max}_{0.002_{NaOMe}}$ 238, 246, 5^{i}, 281 nm. I.r. spectrum: ν OH^{3330} cm^{-1}; ν_{CO1613} cm^{-1}. Both daidzein-7-*O*-benzylether-4′-acetate and daidzein-7-*O*-benzylether afforded daidzein when treated with conc. HCl in acetic acid at 120° for 1 hr as indicated by u.v. and i.r. spectra.

Daidzein-7-*O*-benzylether (60 mg, 0·174 mmole) was stirred at 60° under anhydrous conditions with dry K_2CO_3 (450 mg), acetone (30 ml), MeI (0·01 ml, 0·16 mmole) and MeI-^{14}C (0·47 mg, sp. activity 30 mC/mmole; (Radiochemical Centre Amersham) for 90 min. After the addition of further "cold" MeI (altogether 0·6 ml) the reaction mixture was refluxed for a total of 4 hr. After filtration and evaporation of the solvent, formononetin-7-*O*-benzylether-(methyl-^{14}C) was obtained in quantitative yield. The product was identical in all respects with a sample prepared by benzylation of formononetin with benzyl chloride in acetone in the presence of K_2CO_3. Formononetin-7-*O*-benzyl-ether formed shiny plates from methanol, m.p. 179–180°. U.v. spectrum: λ^{max}_{MeOH} 210, 235, 249, 259, 297, 306 nm unaffected by sodium acetate and sodium methoxide.

Formononetin-7-*O*-benzylether-(methyl-^{14}C) (15 mg) was debenzylated to formononetin-(methyl-^{14}C) by solution in hot acetic acid (4 ml) and heating with conc. HCl (3 ml) at 120° for 35 min. After evaporation of the solvents, the residue was purified on silica gel TLC with (1) benzene–ethyl acetate–MeOH–light petroleum (60–70°), (6:4:1:3;) (2) $CHCl_3$ isopropanol, (10:1); (3) benzene–EtOH, (92:8). Sp. act. 0·547 mC/mmole.

[22] C. D. MILLER, *Planta* **87**, 26 (1969).
[23] G. I. FORREST and D. S. BENDALL, *Biochem. J.* **113**, 757 (1969).
[24] J. BERLIN and W. BARZ, unpublished.

Formononetin-(4-^{14}C)

2,4-Dihydroxyphenyl-4-methoxybenzylketone-(carbonyl-^{14}C)[9] (60 mg) was treated under anhydrous conditions with pyridine (6 ml), piperidine (0·8 ml) and triethyl-ortho-formate (4 ml) at 115° for 22 hr. The dark solution was poured into ice/water, acidified (50% HCl) and continuously extracted with Et_2O for 24 hr. From the Et_2O extract, formononetin was isolated and purified by TLC chromatography as described above. Yield: 42 mg, sp. act. 0·84 mC/mmole.

Daidzein-(4-^{14}C)

Formononetin-(4-^{14}C) (5·3 mg) was demethylated with conc. HI (1·0 ml, sp. gr. 1·6) for 4 hr. at 120°. After dilution with water and decolourization with $Na_2S_2O_5$, the solution was continuously extracted with ether and daidzein purified by TLC with the solvents: $CHCl_3$–isopropanol (10:1), benzene–MeOH (5:1) Yield 4·78 mg, sp. act. 0·87 mc/mmole.

Feeding Experiments

The cultivation of *Cicer arietinum* and *Phaseolus aureus* in hydroponic solution was essentially as described previously.[2] Before each experiment the roots were repeatedly rinsed with sterile water. For all experiments 9-day-old plants were used. For each experiment with formononetin-(methyl-^{14}C), 1·02 mg (2·07 μc) was dissolved in 0·5 ml ethylene glycol mono-methyl ether and added to an appropriate amount of phosphate buffer (0·05 m, pH 8·5). Daidzein-(4-^{14}C), 0·61 mg, 2·1 μc, was administered similarly. $H^{14}CO_3'$ solution (1·5 μc, sp. act. 51 mC/mmole) was placed in a small beaker in the feeding tank and $^{14}CO_2$ evolved by injection of dilute H_2SO_4. The plants were fed in small beakers placed in a large dessicator closed with a flat glass lid and—except for the $H^{14}CO_3'$ experiment—moist air was bubbled through. CO_2 was absorbed in ethylene glycol monomethylether/ethanolamine as described by Jeffay.[25]

Sterile Mung Bean Plants

Batches of 40 H_2O- and EtOH-washed seeds were treated for 20 min with a NaOCl solution (12·5% free chlorine). The seeds were washed free from Cl_2 with sterile H_2O and, under sterile conditions, individually placed in large sterile test tubes (30 × 200 mm) which contained small vials embedded in a layer of nutritional agar (Merck, 2·0%). The test tubes were stoppered with sterile stoppers and kept in a phytochamber under the normal growth conditions.[2] The seeds were given only small amounts of sterile tap water (0·2 ml) as required. 48 hr before the experiments started, the tubes were gently shaken to allow the water to contact the agar. Bacterial contaminations could easily be detected as turbidity on the agar surface.

Fractionation Procedure

After the incubation period the plant material was washed with water, dropped into boiling 70% EtOH and after homogenizing in a Waring blender, repeatedly extracted with hot 70% EtOH. The filtrate was collected, the solvent evaporated under vacuum at 30° and the dry residue thoroughly extracted with light petroleum. After the addition of H_2O (20 ml), phenols were extracted with Et_2O (5 × 50 ml). Hydrolysis of phenolic glycosides was accomplished with 2 N H_2SO_4 at 100° for 2 hr.

The EtOH-insoluble plant material was partly hydrolysed with EtOH–4 N HCl (3:1) at 100° for 4 hr and insoluble material collected by centrifugation.

Isolation of Isoflavonoids

Aliquots of the Et_2O extracts were chromatographed on silica gel TLC with benzene–EtOH (92:8). Formononetin was further purified as mentioned above. The band of biochanin A and homoferreirin was eluted with MeOH and the two compounds separated by paper chromatography with water–isopropanol (4:1). Concentrations were determined spectrophotometrically[1,2] in a Zeiss PMQ II spectrophotometer. All three compounds were diluted with carrier material and purified by repeated crystallizations from MeOH and EtOH and by high vacuum sublimation.

Isolation of Carotenoids

To aliquots of the light petroleum extracts (15 ml), NaOEt (0·1 m, 5 ml) was added and the mixture kept at room temp. for 20 min. After the addition of H_2O (20 ml), carotenoids were extracted with ether–light petroleum (1:1, v/v). The organic layer was washed with H_2O, dried (Na_2SO_4) and concentrated to a small volume. Carotenoids were separated by TLC on cellulose plates which had been impregnated with triglycerides[20] using MeOH–acetone–water (15:5:1) as solvent. The bands of β-cartone, lutein, violaxanthin and neoxanthin were eluted with EtOH and the compounds further purified by two TLC separations on silica gel. For β-carotene light petroleum–benzene (20:1) was used as solvent and for the other three compounds

[25] H. Jeffay and J. Alvarez, *Anal. Chem.* 33, 612 (1961).

light petroleum–acetone (8:5). Absorption spectra were determined in ethanol with a Cary 14 recording spectrophotometer. Extinction coefficients used were as published by Hager.[16] All operations with carotenoids were carried out under dim light.

Radioactivity Assay

Radioactivity of soluble compounds was measured by scintillation spectrometry with a Beckman LS 100 scintillation counter using either dioxane or toluene scintillator. Counting efficiencies were determined by internal standardization (toluene-^{14}C standards). Insoluble material was combusted to CO_2 and counted in the gas phase according to Simon[26] using a Berthold anti-coincidence counter.

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[26] H. SIMON, H. DANIEL and I. F. KLEBE, *Angew. Chem.* 71, 303 (1959).